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JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3600–3607 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.01039-10

Comparison of Two Multilocus Variable-Number Tandem-Repeat Methods and Pulsed-Field Gel Electrophoresis for Differentiating Highly Clonal Methicillin-Resistant *Staphylococcus aureus* Isolates[▽]

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Received 24 May 2010/Returned for modification 2 July 2010/Accepted 2 August 2010

In the United Kingdom, EMRSA-15 and EMRSA-16 account for the majority (~90%) of nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Currently, the standard typing technique, pulsed-field gel electrophoresis (PFGE), is laborious and insufficient for discriminating between closely related subtypes of EMRSA-15 and -16. The objective of the present study was to compare the usefulness of multilocus variable-number tandem-repeat fingerprinting (MLVF) and multilocus variable-number tandem-repeat analysis (MLVA) with PFGE for subtyping these highly clonal MRSA lineages. A panel of 85 MRSA isolates (41 EMRSA-15, 20 EMRSA-16, and 24 MRSA isolates with diverse PFGE patterns) was investigated. In addition, a further 29 EMRSA-15s with identical PFGE patterns from two geographically linked but epidemiologically distinct outbreaks and several sporadic cases were analyzed. PFGE, MLVF, and MLVA resolved 66 (Simpson's index of diversity [SID] = 0.984), 51 (SID = 0.95), and 42 (SID = 0.881) types, respectively, among the 85 MRSA isolates. MLVF was more discriminatory than MLVA for EMRSA-15 and -16 strains, but both methods had comparable discriminatory powers for distinguishing isolates in the group containing diverse PFGE types. MLVF was comparable to PFGE for resolving the EMRSA-15s but had a lower discriminatory power for the EMRSA-16s. MLVF and MLVA resolved the 29 isolates with identical PFGE patterns into seven and six subtypes, respectively. Importantly, both assays indicated that the two geographically related outbreaks were caused by distinct subtypes of EMRSA-15. Taken together, the data suggest that both methods are suitable for identifying and tracking specific subtypes of otherwise-indistinguishable MRSA. However, due to its greater discriminatory power, MLVF would be the most suitable alternative to PFGE for hospital outbreak investigations.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major human pathogen causing considerable morbidity and mortality worldwide. In Scotland and the United Kingdom in general, the incidence of MRSA is high (~35%) with two epidemic clones, EMRSA-15 (ST22) and EMRSA-16 (ST36/ST30), accounting for 70 and 20%, respectively, of isolates referred to the Scottish MRSA Reference Laboratory (SMRSARL). Molecular typing of clinical isolates is important to inform decisions regarding effective control measures. For over a decade, pulsed-field gel electrophoresis (PFGE) of SmaI-digested genomic DNA has been used in United Kingdom reference laboratories for outbreak investigations and epidemiological surveillance, owing to its high discriminatory power and validated interpretation scheme (18). However, it is laborious and time-consuming, with poor interlaboratory comparability, and has no common international nomenclature. Furthermore, ca. 50% of EMRSA-15 strains and 35% of EMRSA-16 strains are indistinguishable by PFGE (4). Accordingly, the method is unsuitable for the tracing of many epidemics caused by EMRSA-15 and EMRSA-16 strains.

Various techniques have been developed to address some of the limitations of PFGE, including *spa* typing and the variable-number tandem-repeat (VNTR)-based methods, multilocus VNTR fingerprinting (MLVF) (15) and multilocus VNTR analysis (MLVA) (6, 13, 16). *spa* typing involves DNA sequencing of the polymorphic VNTR in the 3′ coding region of the *S. aureus*-specific staphylococcal protein A (*spa*) gene (7). The method is more rapid and less laborious than PFGE, and the output is a digital profile, which is easily comparable between laboratories (1). However, it is less discriminatory than PFGE (10, 17) and is unsuitable for investigating the transmission of MRSA in hospitals dominated by EMRSA-15 and EMRSA-16 (8).

MLVF analyzes the variation in the number of tandem repeats in seven genes (*clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE*, *spa*, and *sspA*) by multiplex PCR and has been reported to be highly discrim-

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▽ Published ahead of print on 11 August 2010.

inatory and reproducible (9, 10, 15, 19). Previously, Tenover et al. (19) and Moser et al. (11) demonstrated that MLVF can distinguish between strains with identical PFGE patterns.

MLVA differs from MLVF in that the number of repeats at each locus is determined to produce a numerical profile that can be incorporated into electronic databases and easily shared between laboratories. Although several MLVA schemes have been described, which differ in the loci and PCR protocol used, the MLVA described by Schouls et al. (16) benefits from automated fragment sizing on a DNA sequencer.

To date, the effectiveness of MLVF and MLVA for tracing hospital outbreaks has not been compared. The aim of the present study was to investigate the usefulness of MLVF and MLVA compared to PFGE for subtyping highly clonal EMRSA-15s (ST22) and EMRSA-16S (ST36/ST30) and for tracing hospital outbreaks of infection.

MATERIALS AND METHODS

Strain collection. A total of 114 strains were examined. In order to compare the typeability, discriminatory power, and concordance of the typing methods, two sets of strains ($n = 85$) that had been previously typed by PFGE were used. The first set comprised 59 isolates selected from the database of isolates referred to the SMRSARL for epidemiological typing: 21 EMRSA-15 strains, including the most common PFGE type, A1, and strains that differed from A1 by one band ($n = 4$; R2, R3, R5, and R24); two to three bands ($n = 9$; R6 to R14), four to six bands ($n = 4$; R16 to R19), and seven or more bands ($n = 3$; R21 to R23); 16 EMRSA-16 strains, the most common PFGE type, B1, and strains that differed from B1 by one band ($n = 2$; R32, R33), two to three bands ($n = 5$; R34 to R38), four to six bands ($n = 4$; R39 to R42), and seven or more bands ($n = 4$; R43, R44, R45, and R47); and 22 MRSA strains with diverse PFGE patterns (R49, R51, R52, R54 to R68, and R70 to R73). These strains were not epidemiologically related and were chosen to represent both the diversity of MRSA received by SMRSARL and the breadth of diversity within the EMRSA-15s and -16s. The second set of isolates ($n = 26$; R107 to R132; 20 EMRSA-15, 4 EMRSA-16, and 2 with PFGE type Y) were recovered from two distinct nosocomial outbreaks that were collected over a 1- to 2-month period in 2009.

A third set of isolates ($n = 29$; R74 to R92, R94 to R96, R98 to R100, and R102 to R105) were investigated to determine whether the VNTR methods could distinguish between strains with identical PFGE patterns. The isolates were from two geographically linked but epidemiologically distinct outbreaks and several ($n = 9$) sporadic cases of infection that occurred around the same time. The 29 isolates were from a total of 22 patients and had an indistinguishable EMRSA-15 PFGE profile (A3) but showed differences in antibiotic sensitivity profile.

DNA extraction. Bacterial cultures were subcultured onto Columbia blood agar plates and incubated at 37°C overnight. Five colonies of bacteria were resuspended in lysis buffer (2 mM Tris-EDTA, 1.2% Triton X-100, 30 μg of lysostaphin [Sigma-Aldrich, Poole, United Kingdom]/ml, 5 mg of lysozyme [Sigma-Aldrich]/ml) and incubated for 1 h at 37°C. After lysis, genomic DNA was extracted by using the EasyMag (bioMerieux, Hampshire, United Kingdom), as recommended by the manufacturer. Purified DNA was stored at −20°C.

MLVF. MLVF was performed as previously described by Sabat et al. (15) with some modifications. PCRs contained 1× Qiagen multiplex PCR Mix (Qiagen, Crawley, United Kingdom); ClfA-F, ClfA-R, ClfB-F, ClfB-R, SspA-F, SspA-R, Spa-F, and Spa-R (0.2 μM each); sdrCDE-F and sdrCDE-R (0.5 μM each); and 10 μl of DNA template in a final volume of 50 μl. Amplification was carried out on a thermal cycler (GeneAmp PCR System; Applied Biosystems, Warrington, England, United Kingdom) with an initial denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 30 s, 61°C for 90 s, and 72°C for 120 s, with a final step of 68°C for 15 min. A 10-μl portion of PCR product was mixed with 1 μl of SYBR Safe (Invitrogen, Paisley, United Kingdom) and run on a 2% agarose gel (UltraPure agarose; Invitrogen) with 1% TAE buffer (Sigma-Aldrich) at 100 V for 2.5 h. As a size standard, 1 μl of 100-bp DNA ladder (Invitrogen) at a concentration of 0.5 μg/μl was included in the first and last wells of each gel. Gels were visualized by using a UV transilluminator (GelDoc2000; Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) and stored as TIFF files. Banding patterns were analyzed both visually and by using Bionumerics version 5.10 as described below.

MLVA. MLVA was performed as previously described by Schouls et al. (16) with some modifications. Two multiplex PCRs with different fluorescently labeled primer sets were prepared. Both PCRs contained a 1× Qiagen multiplex PCR mix and 5 μl of DNA template in a final volume of 25 μl. The primer concentrations in Mastermix 1 were 0.4 μM VNTR09_01F-FAM, VNTR09_01R, VNTR61_01F-NED, and VNTR61_01R and 0.2 μM VNTR61_02F-VIC, VNTR61_02R, VNTR67_01F-PET, and VNTR67_01R and in Mastermix 2 were 0.4 μM VNTR21_01F-VIC, VNTR21_1R, VNTR24_01F-PET, and VNTR24_01R and 0.2 μM VNTR63_01F-FAM, VNTR63_01R, VNTR81_01F-NED, and VNTR81_01R. Amplification was carried out on a thermal cycler with an initial denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 90 s, with a final step of 68°C for 15 min.

After the PCR, reactions were diluted 1:50 with nuclease-free water, and 1 μl was mixed with 0.2 μl of LIZ 1200 marker (Applied Biosystems) and 10 μl of HiDi formamide (Applied Biosystems). After heat denaturation at 95°C for 2 min, the fragments were separated on an ABI 3130 DNA sequencer using the FA36_POP7_GS1200 run module. The resulting files were imported and analyzed by using GeneMapper v4.0 software (Applied Biosystems). Loci that did not yield a peak were assigned number 99.

PFGE. PFGE typing of SmaI (Invitrogen)-digested DNA was performed at the Scottish MRSA Reference Laboratory by using a modification of a previously described method (2). Briefly, *S. aureus* colonies from overnight cultures were incorporated into agarose plugs. After bacterial lysis, genomic DNA was digested by using SmaI. PFGE was performed by clamped homogeneous electric field (CHEF) electrophoresis with a CHEF-mapper system (Bio-Rad Laboratories). The fragments were separated with a linear ramped pulse time of 6.8 to 63.8 s over a period of 23 h at 14°C. Gels were analyzed by using DNA analysis software GelCompar II version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice correlation coefficient. A dendrogram was generated by using the unweighted pair group method with arithmetic averages (UPGMA) with a tolerance of 1.5%. PFGE with ApaI was carried out by the same method, except using linear ramped pulse times of 1 to 15 s over a period of 23 h at 14°C. The gels were analyzed by visual inspection.

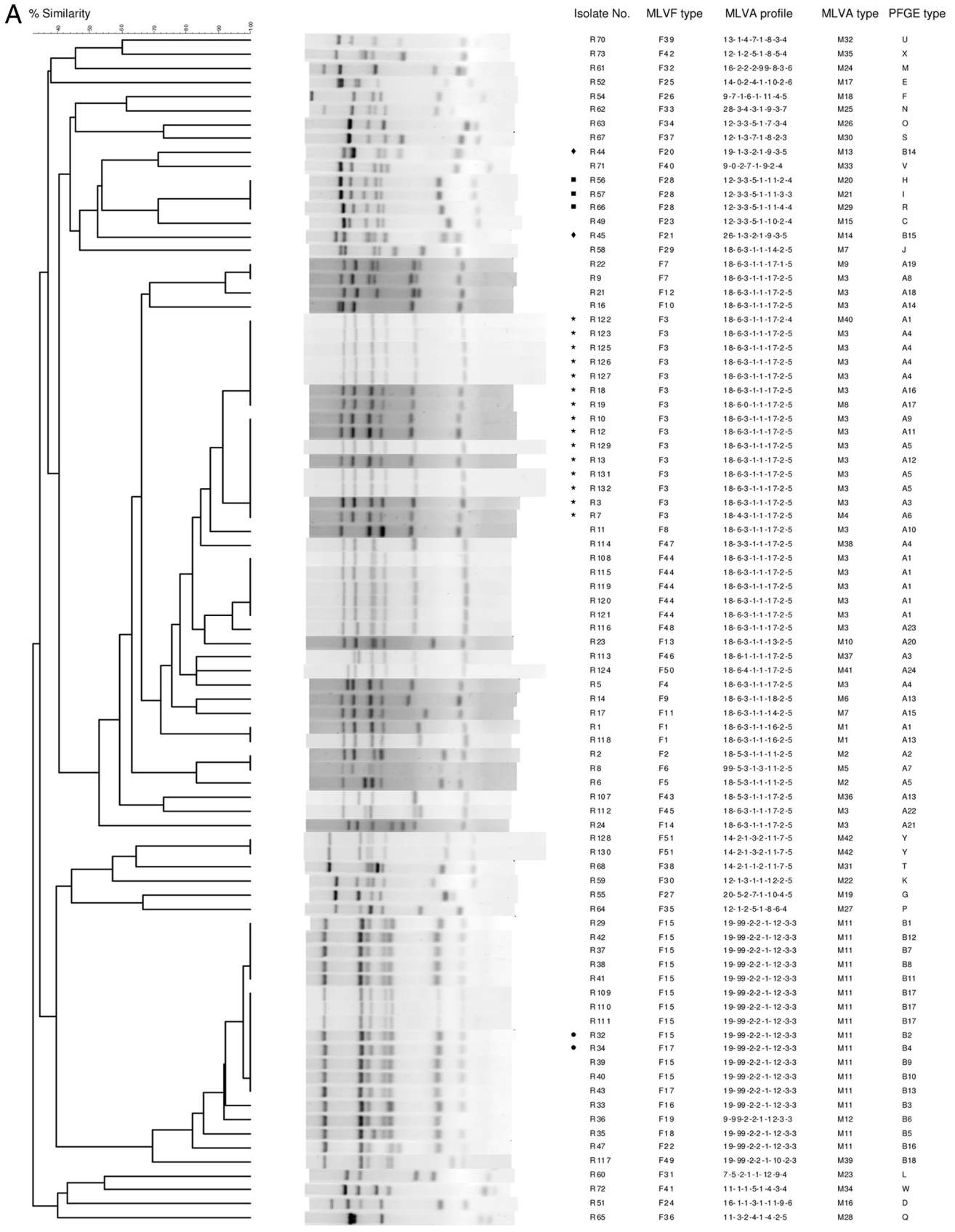
Antibiograms. Antibiograms were determined by using a Vitek GPS-528 card (customized *Staphylococcus* card; bioMerieux).

Data analysis. Bionumerics version 5.10 (Applied Maths) was used to store and analyze the VNTR typing data. MLVF banding patterns were normalized by using the 100-bp DNA ladder, which was loaded in the first and last lanes of all gels. Dendrograms were produced by using Dice coefficients and UPGMA, with position tolerance settings of 1% optimization and 0.75% band position tolerance. For MLVA, repeat numbers were calculated as described by Schouls et al. (16), entered into Bionumerics, and dendrograms were produced by using the categorical distance coefficient and UPGMA.

Ridom EpiCompare (http://www3.ridom.de/epicompare/) was used to calculate the discriminatory power and concordance of the typing methods. V-Dice (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl) was used to measure the variation of the number of repeats at each MLVA locus.

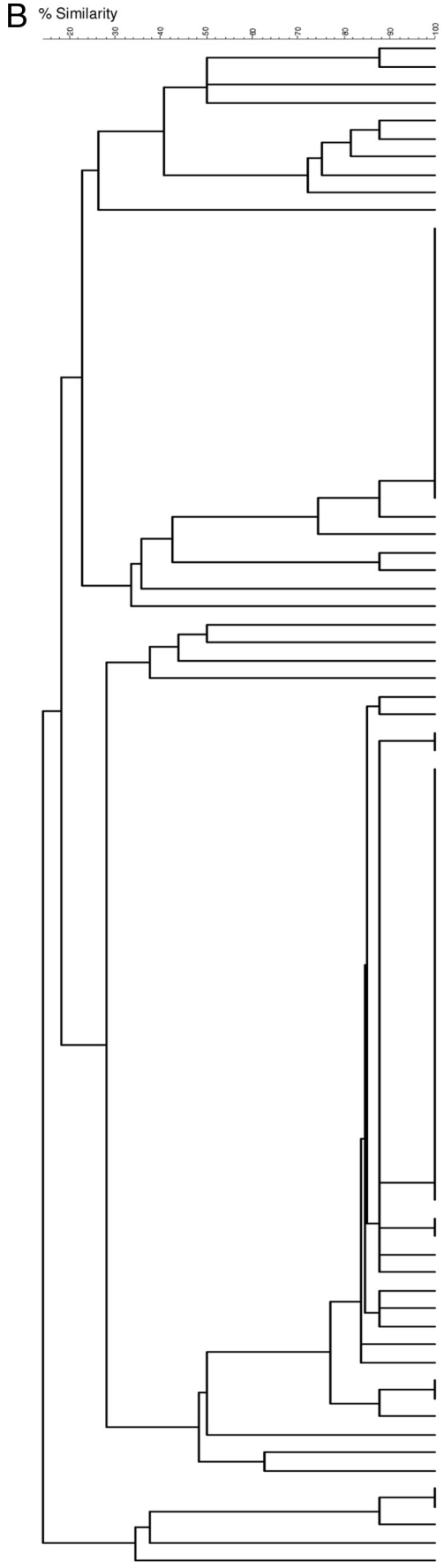
RESULTS

MLVF and MLVA typeability. A typeability of 100% was obtained for MLVF. However, the number of bands varied, between five and seven bands were observed, and this was mostly due to differences in the number of *sdr* genes detected, although in some cases the comigration of bands occurred. A typeability of 76% was obtained for MLVA since an eight-digit profile was obtained for only 65 of the 85 isolates (isolates with ≥1 VNTR loci designated 99 were considered nontypeable). Of the 20 isolates considered nontypeable, 18 (90%) were VNTR61_01 in EMRSA-16 strains. Further analysis of these strains at this locus by single PCR and gel electrophoresis revealed a band of ~2,200 bp, which was not detected by using capillary electrophoresis since it was larger than the upper detection limit of the LIZ marker. BLAST (http://blast.ncbi.nlm.nih.gov/) analysis of the VNTR61-01 primers against the complete genome of MRSA252, a sequenced EMRSA-16 strain, confirmed the primers amplified a region of 2,205 bp.



Isolate No.	MLVF type	MLVA profile	MLVA type	PFGE type
R70	F39	13-1-4-7-1-8-3-4	M32	U
R73	F42	12-1-2-5-1-8-5-4	M35	X
R61	F32	16-2-2-2-99-8-3-6	M24	M
R52	F25	14-0-2-4-1-10-2-6	M17	E
R54	F26	9-7-1-6-1-11-4-5	M18	F
R62	F33	28-3-4-3-1-9-3-7	M25	N
R63	F34	12-3-3-5-1-7-3-4	M26	O
R67	F37	12-1-3-7-1-8-2-3	M30	S
♦ R44	F20	19-1-3-2-1-9-3-5	M13	B14
R71	F40	9-0-2-7-1-9-2-4	M33	V
■ R56	F28	12-3-3-5-1-11-2-4	M20	H
■ R57	F28	12-3-3-5-1-11-3-3	M21	I
■ R66	F28	12-3-3-5-1-11-4-4	M29	R
R49	F23	12-3-3-5-1-10-2-4	M15	C
♦ R45	F21	26-1-3-2-1-9-3-5	M14	B15
R58	F29	18-6-3-1-1-14-2-5	M7	J
R22	F7	18-6-3-1-1-17-1-5	M9	A19
R9	F7	18-6-3-1-1-17-2-5	M3	A8
R21	F12	18-6-3-1-1-17-2-5	M3	A18
R16	F10	18-6-3-1-1-17-2-5	M3	A14
★ R122	F3	18-6-3-1-1-17-2-4	M40	A1
★ R123	F3	18-6-3-1-1-17-2-5	M3	A4
★ R125	F3	18-6-3-1-1-17-2-5	M3	A4
★ R126	F3	18-6-3-1-1-17-2-5	M3	A4
★ R127	F3	18-6-3-1-1-17-2-5	M3	A4
★ R18	F3	18-6-3-1-1-17-2-5	M3	A16
★ R19	F3	18-6-0-1-1-17-2-5	M8	A17
★ R10	F3	18-6-3-1-1-17-2-5	M3	A9
★ R12	F3	18-6-3-1-1-17-2-5	M3	A11
★ R129	F3	18-6-3-1-1-17-2-5	M3	A5
★ R13	F3	18-6-3-1-1-17-2-5	M3	A12
★ R131	F3	18-6-3-1-1-17-2-5	M3	A5
★ R132	F3	18-6-3-1-1-17-2-5	M3	A5
★ R3	F3	18-6-3-1-1-17-2-5	M3	A3
★ R7	F3	18-4-3-1-1-17-2-5	M4	A6
R11	F8	18-6-3-1-1-17-2-5	M3	A10
R114	F47	18-3-3-1-1-17-2-5	M38	A4
R108	F44	18-6-3-1-1-17-2-5	M3	A1
R115	F44	18-6-3-1-1-17-2-5	M3	A1
R119	F44	18-6-3-1-1-17-2-5	M3	A1
R120	F44	18-6-3-1-1-17-2-5	M3	A1
R121	F44	18-6-3-1-1-17-2-5	M3	A1
R116	F48	18-6-3-1-1-17-2-5	M3	A23
R23	F13	18-6-3-1-1-13-2-5	M10	A20
R113	F46	18-6-1-1-1-17-2-5	M37	A3
R124	F50	18-6-4-1-1-17-2-5	M41	A24
R5	F4	18-6-3-1-1-17-2-5	M3	A4
R14	F9	18-6-3-1-1-18-2-5	M6	A13
R17	F11	18-6-3-1-1-14-2-5	M7	A15
R1	F1	18-6-3-1-1-16-2-5	M1	A1
R118	F1	18-6-3-1-1-16-2-5	M1	A13
R2	F2	18-5-3-1-1-11-2-5	M2	A2
R8	F6	99-5-3-1-3-11-2-5	M5	A7
R6	F5	18-5-3-1-1-11-2-5	M2	A5
R107	F43	18-5-3-1-1-17-2-5	M36	A13
R112	F45	18-6-3-1-1-17-2-5	M3	A22
R24	F14	18-6-3-1-1-17-2-5	M3	A21
R128	F51	14-2-1-3-2-11-7-5	M42	Y
R130	F51	14-2-1-3-2-11-7-5	M42	Y
R68	F38	14-2-1-1-2-11-7-5	M31	T
R59	F30	12-1-3-1-1-12-2-5	M22	K
R55	F27	20-5-2-7-1-10-4-5	M19	G
R64	F35	12-1-2-5-1-8-6-4	M27	P
R29	F15	19-99-2-2-1-12-3-3	M11	B1
R42	F15	19-99-2-2-1-12-3-3	M11	B12
R37	F15	19-99-2-2-1-12-3-3	M11	B7
R38	F15	19-99-2-2-1-12-3-3	M11	B8
R41	F15	19-99-2-2-1-12-3-3	M11	B11
R109	F15	19-99-2-2-1-12-3-3	M11	B17
R110	F15	19-99-2-2-1-12-3-3	M11	B17
R111	F15	19-99-2-2-1-12-3-3	M11	B17
● R32	F15	19-99-2-2-1-12-3-3	M11	B2
● R34	F17	19-99-2-2-1-12-3-3	M11	B4
R39	F15	19-99-2-2-1-12-3-3	M11	B9
R40	F15	19-99-2-2-1-12-3-3	M11	B10
R43	F17	19-99-2-2-1-12-3-3	M11	B13
R33	F16	19-99-2-2-1-12-3-3	M11	B3
R36	F19	9-99-2-2-1-12-3-3	M12	B6
R35	F18	19-99-2-2-1-12-3-3	M11	B5
R47	F22	19-99-2-2-1-12-3-3	M11	B16
R117	F49	19-99-2-2-1-10-2-3	M39	B18
R60	F31	7-5-2-1-1-12-9-4	M23	L
R72	F41	11-1-1-5-1-4-3-4	M34	W
R51	F24	16-1-1-3-1-11-9-6	M16	D
R65	F36	11-3-2-4-1-4-2-5	M28	Q

FIG. 1. MLVF (A) and MLVA (B) dendrograms of the 85 MRSA isolates, produced using (i) the Dice similarity coefficient and UPGMA, and (ii) the categorical distance coefficient and UPGMA. PFGE types beginning with an A and B are EMRSA-15s and EMRSA-16s, respectively. Isolates referred to in the results section (R32 and R34; MLVF type F3 isolates; R44 and R45; R56, R57, and R66; and R58) are highlighted with different symbols.



	Isolate No.	MLVF type	MLVA profile	MLVA type	PFGE type
	R64	F35	12-1-2-5-1-8-6-4	M27	P
	R73	F42	12-1-2-5-1-8-5-4	M35	X
	R72	F41	11-1-1-5-1-4-3-4	M34	W
	R70	F39	13-1-4-7-1-8-3-4	M32	U
	R49	F23	12-3-3-5-1-10-2-4	M15	C
	R56	F28	12-3-3-5-1-11-2-4	M20	H
	R66	F28	12-3-3-5-1-11-4-4	M29	R
	R63	F34	12-3-3-5-1-7-3-4	M26	O
	R57	F28	12-3-3-5-1-11-3-3	M21	I
	R62	F33	28-3-4-3-1-9-3-7	M25	N
	R109	F15	19-99-2-2-1-12-3-3	M11	B17
	R110	F15	19-99-2-2-1-12-3-3	M11	B17
	R111	F15	19-99-2-2-1-12-3-3	M11	B17
	R29	F15	19-99-2-2-1-12-3-3	M11	B1
	R32	F15	19-99-2-2-1-12-3-3	M11	B2
	R33	F16	19-99-2-2-1-12-3-3	M11	B3
	R34	F17	19-99-2-2-1-12-3-3	M11	B4
	R35	F18	19-99-2-2-1-12-3-3	M11	B5
	R37	F15	19-99-2-2-1-12-3-3	M11	B7
	R38	F15	19-99-2-2-1-12-3-3	M11	B8
	R39	F15	19-99-2-2-1-12-3-3	M11	B9
	R40	F15	19-99-2-2-1-12-3-3	M11	B10
	R41	F15	19-99-2-2-1-12-3-3	M11	B11
	R42	F15	19-99-2-2-1-12-3-3	M11	B12
	R43	F17	19-99-2-2-1-12-3-3	M11	B13
	R47	F22	19-99-2-2-1-12-3-3	M11	B16
	R36	F19	9-99-2-2-1-12-3-3	M12	B6
	R117	F49	19-99-2-2-1-10-2-3	M39	B18
♦	R44	F20	19-1-3-2-1-9-3-5	M13	B14
♦	R45	F21	26-1-3-2-1-9-3-5	M14	B15
	R61	F32	16-2-2-2-99-8-3-6	M24	M
	R60	F31	7-5-2-1-1-12-9-4	M23	L
	R52	F25	14-0-2-4-1-10-2-6	M17	E
	R71	F40	9-0-2-7-1-9-2-4	M33	V
	R65	F36	11-3-2-4-1-4-2-5	M28	Q
	R55	F27	20-5-2-7-1-10-4-5	M19	G
	R114	F47	18-3-3-1-1-17-2-5	M38	A4
	R7	F3	18-4-3-1-1-17-2-5	M4	A6
	R17	F11	18-6-3-1-1-14-2-5	M7	A15
⊙	R58	F29	18-6-3-1-1-14-2-5	M7	J
	R10	F3	18-6-3-1-1-17-2-5	M3	A9
	R108	F44	18-6-3-1-1-17-2-5	M3	A1
	R11	F8	18-6-3-1-1-17-2-5	M3	A10
	R112	F45	18-6-3-1-1-17-2-5	M3	A22
	R115	F44	18-6-3-1-1-17-2-5	M3	A1
	R116	F48	18-6-3-1-1-17-2-5	M3	A23
	R119	F44	18-6-3-1-1-17-2-5	M3	A1
	R12	F3	18-6-3-1-1-17-2-5	M3	A11
	R120	F44	18-6-3-1-1-17-2-5	M3	A1
	R121	F44	18-6-3-1-1-17-2-5	M3	A1
	R123	F3	18-6-3-1-1-17-2-5	M3	A4
	R125	F3	18-6-3-1-1-17-2-5	M3	A4
	R126	F3	18-6-3-1-1-17-2-5	M3	A4
	R127	F3	18-6-3-1-1-17-2-5	M3	A4
	R129	F3	18-6-3-1-1-17-2-5	M3	A5
	R13	F3	18-6-3-1-1-17-2-5	M3	A12
	R131	F3	18-6-3-1-1-17-2-5	M3	A5
	R132	F3	18-6-3-1-1-17-2-5	M3	A5
	R16	F10	18-6-3-1-1-17-2-5	M3	A14
	R18	F3	18-6-3-1-1-17-2-5	M3	A16
	R21	F12	18-6-3-1-1-17-2-5	M3	A18
	R24	F14	18-6-3-1-1-17-2-5	M3	A21
	R3	F3	18-6-3-1-1-17-2-5	M3	A3
	R5	F4	18-6-3-1-1-17-2-5	M3	A4
	R9	F7	18-6-3-1-1-17-2-5	M3	A8
	R1	F1	18-6-3-1-1-16-2-5	M1	A1
	R118	F1	18-6-3-1-1-16-2-5	M1	A13
	R14	F9	18-6-3-1-1-18-2-5	M6	A13
	R23	F13	18-6-3-1-1-13-2-5	M10	A20
	R113	F46	18-6-1-1-1-17-2-5	M37	A3
	R19	F3	18-6-0-1-1-17-2-5	M8	A17
	R124	F50	18-6-4-1-1-17-2-5	M41	A24
	R122	F3	18-6-3-1-1-17-2-4	M40	A1
	R22	F7	18-6-3-1-1-17-1-5	M9	A19
	R2	F2	18-5-3-1-1-11-2-5	M2	A2
	R6	F5	18-5-3-1-1-11-2-5	M2	A5
	R107	F43	18-5-3-1-1-17-2-5	M36	A13
	R8	F6	99-5-3-1-3-11-2-5	M5	A7
	R59	F30	12-1-3-1-1-12-2-5	M22	K
	R67	F37	12-1-3-7-1-8-2-3	M30	S
	R128	F51	14-2-1-3-2-11-7-5	M42	Y
	R130	F51	14-2-1-3-2-11-7-5	M42	Y
	R68	F38	14-2-1-1-2-11-7-5	M31	T
	R54	F26	9-7-1-6-1-11-4-5	M18	F
	R51	F24	16-1-1-3-1-11-9-6	M16	D

FIG. 1—*Continued.*

TABLE 1. Discriminatory power of MLVF and MLVA compared to PFGE[a]

Group (no. of isolates)	PFGE		MLVF		MLVA	
	No. of types	SID (95% CI)	No. of types	SID (95% CI)	No. of types	SID (95% CI)
EMRSA-15 (41)	25	0.935 (0.889–0.982)	21	0.857 (0.76–0.955)	15	0.632 (0.455–0.808)
EMRSA-16 (20)	18	0.984 (0.952–1.0)	9	0.705 (0.487–0.923)	5	0.368 (0.1–0.637)
Diverse PFGE group (24)	23	0.996 (0.987–1.0)	21	0.986 (0.962–1.0)	23	0.996 (0.987–1.0)
Total (85)	66	0.984 (0.971–0.997)	51	0.95 (0.922–0.979)	42[b]	0.881 (0.827–0.935)

[a] SID, Simpson's index of diversity; CI, confidence interval.
[b] One isolate in the diverse PFGE group had the same profile as one of the EMRSA-15 isolates (R58).

Cluster analysis of MLVF and MLVA data. Cluster analysis of the MLVF and MLVA data are shown in Fig. 1A and B, respectively. Isolates that differed by one band or allele were considered different types. With MLVA, the categorization of different types was straightforward. However, with MLVF, in some cases due to small differences in banding patterns, isolates classified as different types by visual inspection were grouped together by Bionumerics and vice versa. For example, isolates R32 and R34 were considered 100% related according to Bionumerics, but it was possible to see a small shift in one of the *sdr* bands. Also, isolates of MLVF type F3 were split into two closely related clusters according to Bionumerics. Thus, although Bionumerics greatly facilitates the analysis of MLVF data, the results need to be confirmed by visual inspection.

Both VNTR methods clustered the EMRSA-15s together and the EMRSA-16s together, with some exceptions. Two EMRSA-16s (R44 and R45) were not related by MLVF, and only 43% related to the other EMRSA-16s by MLVA. These two isolates belonged to the South West Pacific clone, which are classified as EMRSA-16 (typically ST36-SCC*mec* II) by PFGE but belong to ST30 and carry SCC*mec* IV. Also, one isolate (R58) in the diverse PFGE group clustered with the EMRSA-15s by MLVA. The *spa* type of this isolate was found to be t020, which has previously been associated with EMRSA-15s.

MLVF is more discriminatory than MLVA. Overall, PFGE separated the 85 isolates of MRSA into 66 types (Simpson's index of diversity [SID] = 0.984), where one band difference was considered a different type (Table 1). MLVF produced 51 different banding patterns and had a lower discriminatory index (SID = 0.95) than PFGE. MLVA produced 42 different allelic profiles and had a lower SID (0.881) than both PFGE and MLVF.

MLVF was better able to resolve the EMRSA-15s than MLVA; MLVF produced 21 (SID = 0.857) types compared to 15 (SID = 0.632) by using MLVA. Similarly, within the EMRSA-16s, MLVF had a higher discriminatory power than MLVA. Compared to PFGE, MLVF had a lower but comparable discriminatory power for subtyping the EMRSA-15s, since the 95% confidence intervals overlapped. However, MLVF was less able to resolve the EMRSA-16 strains.

Both methods distinguished between isolates within the group of isolates with diverse PFGE types, with the exception of three isolates (R56, R57, and R66) that were the same by MLVF; these were highly related by MLVA and further analysis by *spa* typing revealed they had the same *spa* type (t008).

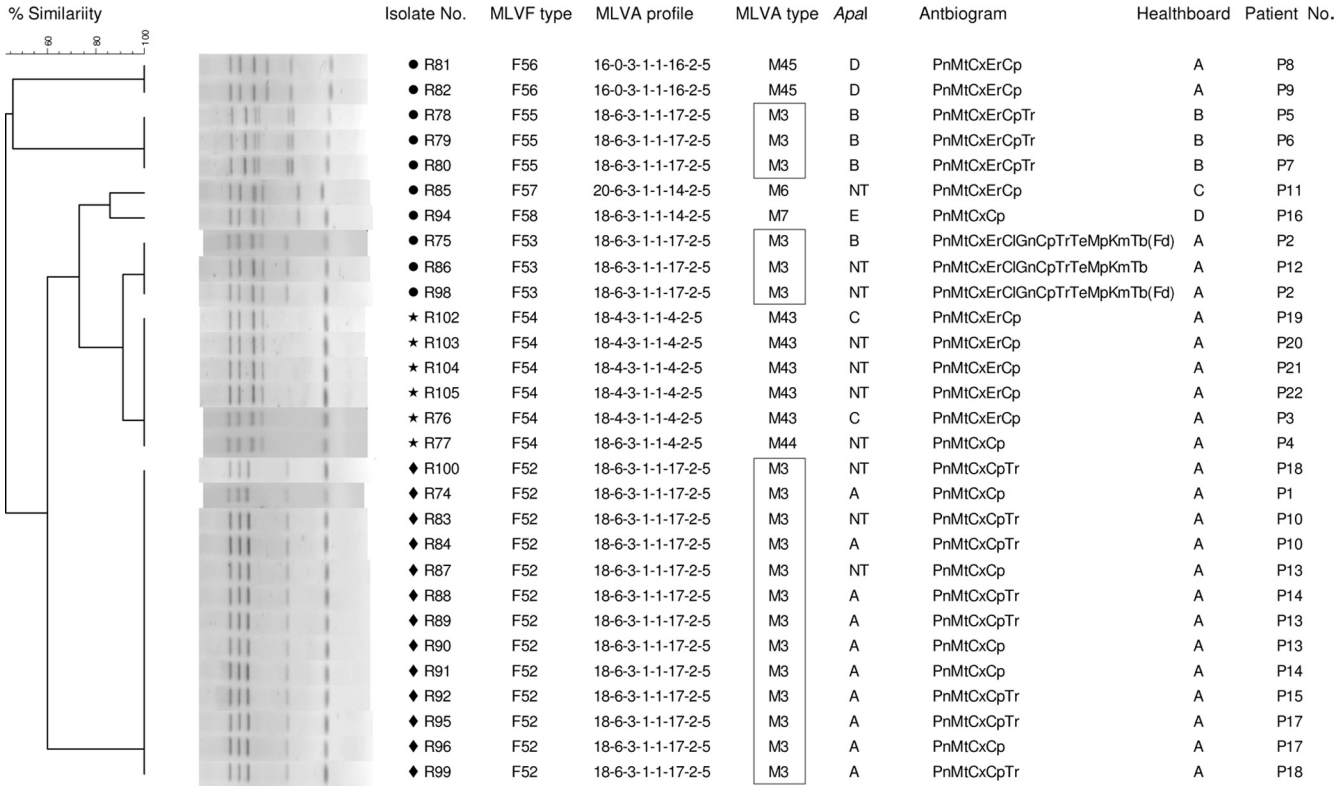
MLVA loci vary in discriminatory potential. For MLVA it was possible to calculate the SIDs for the loci used since the PCR fragments were accurately sized (VNTR24, 0.773; VNTR61_01, 0.748; VNTR09, 0.706; VNTR67, 0.645; VNTR81, 0.580; VNTR61_02, 0.579; VNTR63, 0.578; VNTR21, 0.113). The highest level of diversity was among the repeats of VNTR24, the *spa* locus, whereas the lowest was VNTR21 (noncoding), where a very high level of homoplasy was observed.

Overall, concordance between the methods for subtyping was low. The overall concordance between the typing methods (the probability that similar clustering of isolates would be obtained with the different typing methods) for subtyping was low, with adjusted Rand indices values ranging from 12.1 to 40.4% (Table 2). The highest concordance was between MLVF and MLVA, which is not surprising considering the methods share two common loci. Wallace coefficients were calculated to determine the directional agreement between the typing methods. The results showed two isolates with the same MLVF type had a good probability (75.7%) of having the same MLVA type; however, MLVA was less able to predict MLVF type (31.6%), most likely a reflection of the higher discriminatory power of MLVF. PFGE was reasonably able to predict MLVA (50.9%) and MLVF (61.4%) type, but not vice versa.

MLVA and MLVF can distinguish isolates with identical PFGE patterns. As shown in Fig. 2, MLVF and MLVA separated the 29 PFGE SmaI-indistinguishable isolates into seven and six types, respectively. There was general agreement in subtyping of the isolates by the VNTR methods, although MLVF distinguished the three gentamicin-resistant isolates (R75, R86, and R98), and the three isolates from healthboard B. Most importantly, MLVF and MLVA showed outbreak 1 strains differed from those of outbreak 2, confirming that they were not linked. One exception was isolate R77, which by MLVF was the same as the outbreak 2 strains; however, both the antibiogram (erythromycin-sensitive strain) and the MLVA profile (differed by one allele) suggested it was not linked to outbreak 2. In all cases, multiple samples from the same patient were the same type by MLVF and MLVA. Twenty of these strains were also analyzed by using PFGE ApaI typing, and the results were consistent with those obtained by MLVA (Fig. 2).

TABLE 2. Concordance of MLVF, MLVA, and PFGE for the 85 MRSA isolates

Method	Adjusted Rand index			Wallace coefficient		
	PFGE	MLVF	MLVA	PFGE	MLVF	MLVA
PFGE		0.229	0.121		0.509	0.614
MLVF			0.404	0.164		0.757
MLVA				0.083	0.316	



Isolate No.	MLVF type	MLVA profile	MLVA type	*ApaI*	Antbiogram	Healthboard	Patient No.
● R81	F56	16-0-3-1-1-16-2-5	M45	D	PnMtCxErCp	A	P8
● R82	F56	16-0-3-1-1-16-2-5	M45	D	PnMtCxErCp	A	P9
● R78	F55	18-6-3-1-1-17-2-5	M3	B	PnMtCxErCpTr	B	P5
● R79	F55	18-6-3-1-1-17-2-5	M3	B	PnMtCxErCpTr	B	P6
● R80	F55	18-6-3-1-1-17-2-5	M3	B	PnMtCxErCpTr	B	P7
● R85	F57	20-6-3-1-1-14-2-5	M6	NT	PnMtCxErCp	C	P11
● R94	F58	18-6-3-1-1-14-2-5	M7	E	PnMtCxCp	D	P16
● R75	F53	18-6-3-1-1-17-2-5	M3	B	PnMtCxErClGnCpTrTeMpKmTb(Fd)	A	P2
● R86	F53	18-6-3-1-1-17-2-5	M3	NT	PnMtCxErClGnCpTrTeMpKmTb	A	P12
● R98	F53	18-6-3-1-1-17-2-5	M3	NT	PnMtCxErClGnCpTrTeMpKmTb(Fd)	A	P2
★ R102	F54	18-4-3-1-1-4-2-5	M43	C	PnMtCxErCp	A	P19
★ R103	F54	18-4-3-1-1-4-2-5	M43	NT	PnMtCxErCp	A	P20
★ R104	F54	18-4-3-1-1-4-2-5	M43	NT	PnMtCxErCp	A	P21
★ R105	F54	18-4-3-1-1-4-2-5	M43	NT	PnMtCxErCp	A	P22
★ R76	F54	18-4-3-1-1-4-2-5	M43	C	PnMtCxErCp	A	P3
★ R77	F54	18-6-3-1-1-4-2-5	M44	NT	PnMtCxCp	A	P4
♦ R100	F52	18-6-3-1-1-17-2-5	M3	NT	PnMtCxCpTr	A	P18
♦ R74	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCp	A	P1
♦ R83	F52	18-6-3-1-1-17-2-5	M3	NT	PnMtCxCpTr	A	P10
♦ R84	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P10
♦ R87	F52	18-6-3-1-1-17-2-5	M3	NT	PnMtCxCp	A	P13
♦ R88	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P14
♦ R89	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P13
♦ R90	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCp	A	P13
♦ R91	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCp	A	P14
♦ R92	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P15
♦ R95	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P17
♦ R96	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCp	A	P17
♦ R99	F52	18-6-3-1-1-17-2-5	M3	A	PnMtCxCpTr	A	P18

FIG. 2. MLVF dendrogram of the 29 EMRSA-15 isolates indistinguishable by PFGE using SmaI. The dendrogram was produced using the Dice similarity coefficient and UPGMA. Outbreak 1, outbreak 2, and isolates from sporadic cases are denoted by diamonds, stars, and circles, respectively. NT, not tested. Antibiogram abbreviations: Pn, benzylpenicillin; Mt, methicillin; Cx, cefuroxime; Er, erythromycin; Cp, ciprofloxacin; Cl, clindamycin; Gn, gentamicin; Te, tetracycline; Mp, mupirocin; Km, kanamycin; Tb, tobramycin; Fd, sodium fucidate.

DISCUSSION

Several different VNTR-based schemes have been described for genotyping *S. aureus*; however, there is no consensus on the best method or set of markers to use. This is the first study to compare two different VNTR methods, MLVF (15) and MLVA (16). The methods were chosen since they both have attractive features. MLVF is rapid and cheap, while MLVA involves automated fragment sizing and produces unambiguous data that can be easily shared between laboratories.

The aim of the present study was to compare the usefulness of MLVF and MLVA for subtyping EMRSA-15 and EMRSA-16 strains, the predominant lineages of MRSA circulating in Scotland and the rest of the United Kingdom. Therefore, our strain collection included isolates with various degrees of genetic relatedness, as defined by PFGE, within these two highly clonal lineages. The results showed MLVF had a greater discriminatory power than MLVA, since MLVF was better able to distinguish between subtypes of EMRSA-15 and EMRSA-16; both methods were highly discriminatory for distinguishing between isolates in the group of diverse PFGE strains.

Compared to PFGE, the VNTR methods had lower discriminatory powers, especially MLVA. Previously, Schouls et al. (16) showed MLVA was comparable to PFGE for genotyping a large collection of diverse *S. aureus* isolates, the majority (78.1%) of which were MRSA. The discrepancy in the results may be explained by differences in the strain collections used. Schouls et al. (16) showed the diversity indices differed between MLVA complexes (MCs). Most importantly, they found MC22 (= CC22, EMRSA-15s), which mostly consisted of MRSA strains (92.1%), had a relatively low diversity index (76.8%), along with MC398 (72.1%) and MC80 (67.1%). Since our strain collection consisted of a large proportion (48%) of EMRSA-15s, this may partly explain the lower discriminatory power observed in our study. Interestingly, MC30 (= CC30) had a high diversity index of 95.5%, whereas we found MLVA had a particularly low SID for the EMRSA-16s. However, a large proportion of the MC30 isolates were MSSAs and/or Panton-Valentine leukocidin-positive and thus most likely belonged to ST30 rather than to ST36. In our study, MLVA differentiated between the two ST30 isolates.

Another explanation for the relatively lower discriminatory power obtained for the VNTR methods compared to previous reports (9, 10, 15, 16) is the way the isolates were selected. Although epidemiologically related isolates from two hospital outbreaks were included in our evaluation panel, a larger percentage (69%) of the isolates were chosen based on differences in PFGE, thus introducing selection bias. Previously, Luczak-Kadluboska et al. (9) reported MLVF was as least as discriminatory as PFGE when isolates had not been preselected. Interestingly, in the present study, MLVF was comparable in discriminatory power for subtyping the EMRSA-15s (at the 95% confidence level) but was less able to resolve the EMRSA-16 strains, which is consistent with a previous study.

Hardy et al. (5) reported staphylococcal interspersed repeat unit (SIRU) typing was better able to resolve subtypes of EMRSA-15 compared to those of EMRSA-16.

The differences in discriminatory power of the methods used in the present study are primarily due to the different loci used in the assays. Different tandem repeats are known to vary at different rates (12). The methods used here share two common loci, *sspA* and *spa*. So, the higher resolving power of MLVF was associated with one or more of the other loci (*clfA*, *clfB*, and/or *sdrCDE*). In a recent report, Rasshaert et al. (14) showed *clfA*, *clfB*, and *sdrCDE*, as well as *spa*, were the only discriminatory loci among a panel of 15 loci evaluated to subtype isolates of MRSA ST398.

Calculation of the diversity indices for the MLVA loci showed VNTR24 (*spa* locus) was the most polymorphic, while VNTR21 (noncoding) was the least variable between strains. This finding supports those reported by Schouls et al. (16), and Hardy et al. (5) also found the *spa* locus to be the most variable among the SIRU loci used in their MLVA scheme. In the present study, obtaining such a low diversity index for VNTR21 (noncoding) suggests this should be replaced with a more polymorphic VNTR. Similarly, amplification of VNTR61_01 in EMRSA-16s produced a band too large for detection by capillary electrophoresis, limiting the usefulness of this locus since two isolates showing no amplification are considered identical.

Data analysis for MLVA was straightforward since the method is automated and unambiguous profiles are obtained. However, MLVF data analysis is more subjective. Bionumerics greatly facilitated the analysis of the MLVF banding patterns; however, visual inspection was necessary to confirm the results due to small band shifts. Previously Rasshaert et al. (14) used visual categorization rather than Bionumerics to identify subtypes of ST398 genotyped by using a fingerprinting method very similar to MLVF. The subjectivity in MLVF data analysis restricts the utility of the method.

The concordance data showed there was low concordance between the VNTR methods and PFGE at the subtyping level; however, cluster analysis showed the majority of the EMRSA-15s clustered together, as did the EMRSA-16s, showing greater concordance at the group level. Previously, Hardy et al. (5) showed the clustering of these epidemic strains was the same by SIRU typing and PFGE; however, subtyping differed between the two. Other studies comparing VNTR methods with PFGE have shown much greater concordance between methods at the group level compared to the subtyping level (3, 10).

Although MLVA had a lower discriminatory power than MLVF in the technical assessment, both methods were able to distinguish between strains with identical PFGE patterns, so would be useful for tracing the spread of certain subtypes. Previously Tenover et al. (19) and Moser et al. (11) showed MLVF could distinguish between isolates with identical PFGE types. In the present study, we also showed that the use of a different PFGE restriction enzyme, ApaI, discriminated between strains indistinguishable using SmaI. ApaI has recently been used for subtyping animal-associated ST398 isolates that are not typeable by SmaI (14). Similarly, *spa* typing was able to discriminate between the same PFGE subtypes, and they clustered in the same way as MLVA (data not shown).

In conclusion, there is a need to replace PFGE with a more discriminatory and convenient method. MLVF and MLVA compare favorably with PFGE with regard to speed and simplicity. In the present study we were particularly interested in investigating the ability of MLVF and MLVA to subtype EMRSA-15 and EMRSA-16, which are the predominant strains circulating in United Kingdom hospitals. We found both methods would be suitable for identifying and tracking specific subtypes of MRSA. MLVA was convenient as it generated unambiguous, easily comparable profiles. However, optimization of the markers is required to improve the discriminatory power of the method. Due to its greater discriminatory power, MLVF would be more suitable than MLVA for hospital outbreak investigations.

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